

Docket No.: 17563/004001
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Kazuhiro Kukae

Application No.: 10/544,212

Confirmation No.: 8443

Filed: August 2, 2005

Art Unit: 1651

For: PROCESS FOR PRODUCING SUGAR CHAIN
ASPARAGINE DERIVATIVE Examiner: K. Ariani

DECLARATION BY KAZUHIRO FUKAE UNDER 37 C.F.R. § 1.132

MS RCE
Commissioner for Patents
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Dear Madam:

I, Kazuhiro FUKAE, a Japanese national, having an address at c/o OTSUKA CHEMICAL CO., LTD., 463, Kagasuno, Kawauchi-cho, Tokushima-shi, Tokushima-ken 771-0193 Japan, declare that:

1. I am an inventor of the above-identified application.
2. I have studied the Office Action dated March 18, 2010.
3. I have conducted the following experiments to demonstrate superiority of the present invention over the cited reference.

EXPERIMENTS

I conducted experiments on one Example (Example 1) and one Comparative Example, as described below.

Example 1

One egg yolk was placed as broken into 67ml of ethanol (EtOH) being stirred. The mixture was stirred for about 5 hours and then filtered, followed by washing with 30 ml of EtOH. To the resulting residue was added 83 ml of EtOH, and the mixture was stirred overnight. The mixture was thereafter filtered, followed by washing with 30 ml of EtOH. The residue thus obtained was dried, giving about 3 g of delipidated egg yolk.

(a) The delipidated egg yolk was dissolved in a phosphate buffer (7.0 in pH, 30 ml), and NaN₃ (10 mg) was added to the solution. Orientase ONS (product of Hankyu Bioindustry Co., Ltd., 1.0 g) was added to the solution, and the mixture was allowed to stand at 50 °C for about 24 hours. After the termination of the reaction was confirmed by TLC, the reaction mixture was filtered with Celite. The filtrate was concentrated and purified by gel filtration column chromatography (Sephadex G-25, 2.5 × 100 cm, H₂O). The fractions containing the desired saccharides were collected, concentrated and then freeze-dried.

(b) To the residue (about 430 mg) thus obtained were added Tris-hydrochloric acid/calcium chloride buffer solution (7.5 in pH, 43 ml) and NaN₃ (21 mg) to obtain a solution. Actinase E (43 mg) was added to the solution, and the mixture was allowed to stand for 24 hours while pH was checked every 12 hours. Another portion of actinase E (21.5 mg) was added to the reaction mixture 24 hours later, followed by further reaction for about 48 hours while pH was checked. After the termination of the reaction was confirmed by TLC, the reaction mixture was filtered with Celite, and the filtrate was concentrated and purified by gel filtration column chromatography (Sephadex G-25, 2.5 × 100 cm, H₂O). The fractions containing the desired saccharides were collected, concentrated and then freeze-dried.

(c) The residue (about 120 mg) thus obtained was dissolved in 1.5 ml of water, and 26 mg of sodium bicarbonate was added to the solution. To the mixture was added a solution of 68 mg of Fmoc-Osu (9-fluorenylmethyl succinimidyl carbonate) in 2.5 ml of dimethylformamide, and the resulting mixture was reacted at room temperature for 2 hours. After the disappearance

of the starting material was confirmed by TLC (isopropanol: 1M aqueous solution of ammonium acetate = 3:2), the reaction mixture was concentrated by an evaporator. To the residue were added 15 ml of water and 25 ml of diethyl ether, and the mixture was stirred for 10 minutes, followed by a extraction procedure. The aqueous layer was further washed with 15 ml of diethyl ether, and thereafter concentrated and freeze-dried. The product was purified using an ODS column (Wako-Gel 100C18) for gradient elution. The fractions containing oligosaccharides were collected, concentrated and freeze-dried.

(d) The residue was purified by an HPLC fractionating column (YMC-Pack R&D ODS, D-ODS-5-A, 20 × 250mm, AN/25 mM AcONH₄ buffer = 20/80, 7.5 ml/min., wavelength 274 nm). A fraction of main peak eluted about 15 minutes later was collected, then concentrated and desalted on an ODS column. After freeze-drying, the product was obtained about 13.3 mg of the desired disialo Fmoc oligosaccharide derivative.

Comparative Example

The same procedure was conducted as in Example 1, except that the filtrate of the reaction mixture after reaction with Orientase was not purified by gel filtration column chromatography in step (a) which corresponds to the isolating step (b) in Claim 14. The detailed procedure is as follows.

One egg yolk was placed as broken into 67ml of ethanol (EtOH) being stirred. The mixture was stirred for about 5 hours and then filtered, followed by washing with 30 ml of EtOH. To the resulting residue was added 83 ml of EtOH, and the mixture was stirred overnight. The mixture was thereafter filtered, followed by washing with 30 ml of EtOH. The residue thus obtained was dried, giving about 3 g of delipidated egg yolk.

(a) The delipidated egg yolk was dissolved in a phosphate buffer (7.0 in pH, 30 ml), and NaN₃ (10 mg) was added to the solution. Orientase ONS (product of Hankyu Bioindustry Co.,

Ltd., 1.0 g) was added to the solution, and the mixture was allowed to stand at 50 °C for about 24 hours. After the termination of the reaction was confirmed by TLC, the reaction mixture was filtered with Celite. The filtrate was concentrated and then freeze-dried.

(b) To the residue (about 1.44 g) thus obtained were added Tris-hydrochloric acid/calcium chloride buffer solution (7.5 in pH, 144 ml) and NaN₃ (72 mg) to obtain a solution. Actinase E (144 mg) was added to the solution, and the mixture was allowed to stand for 24 hours while pH was checked every 12 hours. Another portion of actinase E (72 mg) was added to the reaction mixture 24 hours later, followed by further reaction for about 48 hours while pH was checked. After the termination of the reaction was confirmed by TLC, the reaction mixture was filtered with Celite, and the filtrate was concentrated and purified by gel filtration column chromatography (Sephadex G-25, 2.5 × 100 cm, H₂O). The fractions containing the desired saccharides were collected, concentrated and then freeze-dried.

(c) The residue (about 1.72 g) thus obtained was dissolved in 21.4 ml of water, and 372 mg of sodium bicarbonate was added to the solution. To the mixture was added a solution of 972 mg of Fmoc-Osu (9-fluorenylmethyl succinimidyl carbonate) in 30 ml of dimethylformamide, and the resulting mixture was reacted at room temperature for 2 hours. After the disappearance of the material was confirmed by TLC (isopropanol: 1M aqueous solution of ammonium acetate = 3:2), the reaction mixture was concentrated by an evaporator. To the residue were added 214 ml of water and 357 ml of diethyl ether, and the mixture was stirred for 10 minutes, followed by a extraction procedure. The aqueous layer was further washed with 186 ml of diethyl ether, and thereafter concentrated and freeze-dried. The product was purified using an ODS column (Wako-Gel 100C18) for gradient elution. The fractions containing oligosaccharides were collected, concentrated and freeze-dried.

(d) The residue was purified by an HPLC fractionating column (YMC-Pack R&D ODS, D-ODS-5-A, 20 × 250mm, AN/25 mM AcONH₄ buffer = 20/80, 7.5 ml/min., wavelength 274 nm). A fraction of main peak eluted about 15 minutes later was collected, them concentrated and

desalted on an ODS column. After freeze-drying, the product afforded about 7.63 mg of the desired disialo Fmoc oligosaccharide derivative.

RESULT

The sugar chain asparagine derivatives obtained in Example 1 was 13.3 mg, whereas that obtained in Comparative Example was 7.63 mg. That is, the yield of Example 1 is about 75% more than that of the Comparative Example. In addition, the Comparative Example needed the increased amount of agent in the subsequent steps, which was highly inefficient. Thus, this experimentation clearly shows that the purification (isolation) step by gel filtration column chromatography is important to obtain the high yield, and a method of the invention produces substantially improved results, as compared with a conventional method without a purification (isolation) step.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed on: July 31, 2010



Kazuhiro FUKAE